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Detecting Adreno-Cortical Activity in Gorillas: A Comparison of Faecal Glucocorticoid Measures Using RIA Versus EIA

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Abstract: The conservation community is concerned that the remnants of highly endangered Mountain gorilla are being exposed to enormous stressors in their habitat but no assay has been validated to monitor stress markers in their fecal medium. Non specific ICN Corticosterone RIA and Munro Cortisol EIA were validated for measurement of free-ranging Mountain gorilla Faecal Glucocorticoids Metabolites (FGM) to non-invasively detect effect of environmental stressors on the adrenocortical activity in gorillas. Parallelism and quantitative recovery of FGM showed that the RIA and EIA can reliably measure corticoid metabolites in this medium. High Performance Liquid Chromatography confirmed presence of FGM in faecal extracts. Peak immunoreactive metabolites constituting 35.43% of FGM measured by the RIA co-eluted with maximum corticosterone radioactivity, the EIA measured multiple immunoreactive peak metabolites which were less polar than the cortisol, corticosterone and DOC standard hormones. For Lowland gorilla, peak FGM eluted at fractions 27 and 53 under the 100% Isocratic gradient (San Diago protocol), and 20-80% Methanol gradient (National Zoo protocol) respectively; while peak FGM eluted at fraction 8 and 47 in Mountain gorilla under 100% Isocratic and 20-80% Methanol gradients respectively. After injection of Lowland gorilla with long acting Adrenal Cortico-trophic Hormone (ACTH) gel (150U Acthar Gel), urinary cortisol increased by 5-fold ($p<0.05$) within the first 6 h and then decreased to original values by 24 h. FGM were significantly elevated ($p<0.05$) between 72 and 96 h (RIA) and between 48 and 120 h (EIA) and there after decreased towards the pre-ACTH levels. Both assays detected a 2-fold increase in FGM 48 h post ACTH. Mean pre-ACTH and post-ACTH FGM amounts measured by RIA were 36 times ($p<0.05$) more than those measured by the EIA. For field application FGM were measure from three free-ranging Mountain gorilla individuals before and after chasing the gorilla family from fields. Between the 2nd and 3rd day of chase, FGM (RIA) increased by 44, 21 and 71% in the silverback, adult female and infant respectively. We conclude that measurement of FGM using ICN RIA and Munro Cortisol EIA offers a practical promise to non-invasively monitor adreno-cortical activity in gorillas.

Key words: ACTH challenge, captive lowland gorilla, chasing gorillas, faecal glucocorticoid metabolites, free-ranging mountain gorilla, validation

INTRODUCTION

Although there is no single definition or physiological measure of 'stress', glucocorticoid hormone (e.g., cortisol, corticosterone) secretion has been considered reflective of adrenal status in many species held *ex situ* as well as those living *in situ* (Hofer and East, 1998). External stressors are known to activate the Hypothalamic-Pituitary-Adrenal (HPA) axis resulting in release of glucocorticoids (Bradley *et al*., 1980; Sapolsky, 1985; Alexander and Irvine, 1998). There exists physiological feedbacks that regulate the glucocorticoid

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production, however, chronic stress can override these regulatory mechanisms resulting in prolonged adrenal stimulation, which ultimately can have deleterious consequences, eventually suppressing growth and reproduction, compromising the immune function, leading to pathology (Rilley, 1981; Moberg, 1985) and eventually having profound effects on the species' population demographic dynamics and survival.

Monitoring health status is important for effective management of captive and free-ranging wildlife and non-invasive methods have been developed to facilitate assessment of reproductive and stress physiology without the adverse effects of instituting chemical or physical restraints. The broad application of these methods to a wide range of species is related to the conservation of the parent steroid hormone molecule during metabolism across taxa and the stability of resultant steroid hormone metabolites in excreta (Lasley and Kirkpatrick, 1991).

The elaborate biochemical basis of steroidogenesis and break down were compiled by Harper (1969) and Grant (1974). Furthermore, other specific studies have revealed that steroids vary in the extent to which they are metabolized before excretion and that proportions of steroid metabolites excreted in urine or faeces usually is species or taxon specific. For example, most felids excrete >90% of gonadal steroids into faeces (Brown *et al*., 1994), whereas baboons excrete >80% of gonadal steroids into urine (Wasser *et al*., 1991). Thus there can be differences in hormones measured between media, but the trends in each medium (urine or faeces) can be reflective of the prevailing physiological status in the animal.

Although it is possible to monitor the reproductive and adrenocortical hormonal profiles in the animal excreta, it is currently difficult to compare values of hormone concentrations among laboratories because of the differences that originate from varying technical procedures, cross-creativities used in immunoassays; in addition to hitherto un-standardized field sampling (e.g., sample age and preservation types), (Desaulniers *et al*., 1989; Hultén *et al*., 1995; Schwarzenberger *et al*., 1996b; Palme *et al*., 1997; Millspaugh and Washburn, 2004). Nonetheless, carefully designed and validated studies within laboratories contribute enormously to the body of knowledge for understudied species.

The Mountain gorilla is highly endangered and threatened with extinction. With an estimate of about 720 living in the wild (WCS, 2007), and none in captivity, its survival is dependent on the quality of conservation measures aimed at protecting their habitat, animal health and psychological well-being. Human-induced environmental changes to the Mountain gorilla habitat are adversely impacting their chances for survival. The conservation community has raised its concern over the pressure being put on the gorillas (Butynski and Kalina, 1998); and such pressure may manifest as animal "stress", which has the potential to cause adverse health consequences (Cohen and Crnic, 1983; Breazile, 1987), negatively impacting reproductive fitness, and ultimately the species' survival (Goncharov *et al*., 1979; Sapolsky, 1987; Johnson *et al*., 1991; Norman, 1993; Cameron, 1997; Bahr *et al*., 1998).

Because about one-half (340) of all remaining Mountain gorillas inhabit a small habitat in Bwindi Impenetrable National Park (BINP) (WCS, 2007; Gray *et al*., 2007), research designed to assess the impact of environmental change on gorilla well-being has been made a priority. Decision-makers require scientific data for design of effective conservation strategies that will ensure the long-term survival of Mountain gorillas. The BINP Management Plan specifies that policies should "ensure that necessary behavioural, physiological and ecological monitoring is carried out on gorilla groups to assess the impact of tourism and other programs (human activity) on Bwindi gorillas, and to ensure that adequate research is done on the gorillas and local people, and possibly on domestic animals" (Uganda National Parks, 1995).

Non-invasive behavioural and endocrine methods permit an objective assessment of the impact of social and human-related disturbances. Such data can help to develop a baseline of information about the fitness of free-living Mountain gorillas when combined with additional measures of animal health, including body condition, parasite loads and reproductive success. Taken together, the data can provide the first information needed by the park authority to begin making objective decisions on the health and welfare management of the free-ranging habituated Mountain gorillas.

Since the adrenal gland plays a major physiological role to an animal's response to stressors, it is important to have a non-invasive tool with which to monitor the adrenal activity as an index of stress physiology. Authors who have reviewed use of the non-invasive endocrine measures of reproduction and stress in wild populations have noted that the advantage of using excreta is that; pooling of urine (in the bladder) or faeces (in the intestinal tract) dampens episodic secretory patterns that normally occur in blood circulation, thereby producing hormonal patterns that accurately reflect physiological status, and additionally in contrast to blood (where only a few samples usually are recoverable); non-invasive endocrine methods allow frequent (even daily) sampling thereby permitting longitudinal studies while enhancing statistical power, and all this is achieved without disturbing the animals which otherwise can in itself elicit stress (Lasley and Kirkpatrick, 1991; Brown *et al*., 1994; Hodges, 1996; Schwarzenberger *et al*., 1996a, 1997; Whitten *et al*., 1998; Monfort, 2001).

A technique to measure urinary cortisol in free-ranging gorillas was validated by Robbins and Czekala (1997), however, this approach has some limitations due to the fact that urine samples are difficult to obtain from free-ranging gorillas without being intrusive. This makes it hard for Mountain gorilla conservation agencies in host countries to design a health-monitoring program for the gorilla population based on this technique. An alternative method is the evaluation of adrenal glucocorticoids in gorilla faeces; which are easier to collect compared to urine.

The objectives of this study included i) validating a Radio-Immunoassay (RIA) and an Enzyme Immunoassay (EIA) for detecting glucocorticoids in faeces from gorillas; ii) comparing 2 faecal glucocorticoid chromatography procedures on gorilla faecal extracts; iii) testing whether faecal glucocorticoids increase after challenge with Adrenocorticotrophic Hormone (ACTH) and establishing the excretory lag-time; iv) correlating glucocorticoid levels between urine and faeces; and v) testing the field applicability of the assay to document stress resulting from chasing free-ranging gorillas by humans.

MATERIALS AND METHODS

Study animals: The current study was part of a large project carried out between 2000 and 2005. Animals involved in the assay validations included three adult male captive western lowland gorillas (Gorilla gorilla gorilla) resident at St Louis Zoo, MO, USA (n = 2) and Mexico City Zoo, Mexico (n = 1) and one adult female resident at Brookfield Zoo, Il. USA (n = 1) were housed individually for administration of an ACTH challenge test. For field application, one adult male, one adult female and one juvenile male/female free ranging Mountain gorillas in a habituated family group in the Mubale area of Bwindi Impenetrable National Park (BNIP), South Western Uganda, were studied. Permission to collect faecal samples from the Mountain gorillas was obtained from the Uganda Wildlife Authority (UWA). Export and Import permits for Mountain gorilla fecal samples was obtained from the Uganda Council of Science and Technology (UNCST) and CITES Export license (No.167) given by the CITES management authority (The Assistant Commissioner Wildlife, Ministry of Tourism Trade and Industry) in Uganda after presenting a CITES import permit (No. 00US772163/9) licensed to the Morris Animal Foundation by the U.S. Fish and Wildlife Service, Department of Interior (USA).

Study design, sample collection and processing:
Lowland gorillas: Measurement of glucocorticoid levels in urine and faeces to indicate adrenal response in Lowland gorillas was validated by administering a bolus injection of long acting ACTH gel (150 IU Acthar Gel, Rhone-Poulenc Rorer Pharmaceuticals, Inc., Collegeville, Pennsylvania 19034, USA,) together with 25 mg of cortisol (combined with 10 mg of deuterium-labelled cortisol) intramuscularly by blow dart. Urine and faeces were collected for 3 days before ACTH administration to establish baseline adrenal corticoid concentrations. Following ACTH injection, all urine and faeces were collected in individually labelled vials and plastic tubes respectively for 4 days and frozen. Samples were shipped on ice; urine was shipped to San Diego (California, USA) and faeces sent to the Smithsonian Institution's Centre for Conservation and Research (CRC), (Virginia; USA) for analysis.

Faecal samples were dried in a Speedvac Rotary Evaporator (Forma Scientific, Marietta, Ohio, USA), pulverized and stored at -20^{o}C until extraction and analysis. Detection and quantification of the FGM was achieved by assaying the faecal extracts using ICN corticosterone RIA and Munro cortisol EIA following the manufacturer's protocol.

Urine was processed and analyzed as described by Czekala *et al*. (1994). Urine was hydrolyzed by using 0.1 mL urine, 0.4 ml pH 5 phosphate buffer, and 0.02 ml glucuronidase - arylsulfatase (Boehringer Mannheim, Indianapolis, IN) and then incubated in a 37^{0}C water bath overnight. Glucocorticoids were extracted by using 5 mL anhydrous diethylether, after which they were re-constituted in 1.0 mL pH 7 phosphate buffer. The extracts were assayed by incubating 0.05 ml of the reconstituted sample (made up to 0.5 ml with buffer) with 5,000 c.p.m. ^{3}H-Cortisol, and Cortisol 21-BBSA - hemisuccinate antiserum (1:3000, F39 Yen). Following overnight incubation at 4^{0}C, separation of antibody bound and free-steroid was achieved by addition of 0.2 ml charcoal-dextran mixture (0.625 and 0.625%, respectively in phosphate buffer. The variation in urine concentration was corrected for by dividing creatinine levels in each sample into hormonal concentration (Taussky, 1954). To eliminate overly dilute specimens, urine samples with creatinine concentrations of less than 0.05 mg/ml were excluded from the analysis (Czekala and Robbins, 1997).

Field application of FMG measures in Mountain gorillas: Investigation of utility of FGM measures to detect adrenal response to exogenous environmental stressors in free-ranging Mountain gorillas was achieved by taking faecal samples of a dominant silverback, an adult female and an infant of Mubale family; before and after an observed event that was deemed to be 'stressful' for the individuals, specifically 3 days before up to 5 days after the family was chased from a banana plantation in January 2002. Gorilla tracking began at 8.00 am from ranger post to the area where the family had been left feeding the previous day. The gorilla trail was followed till the nesting site was reached. The individual's night nest was identified with the help of guides/trackers using

nesting patterns and confirmation made by recovering silver hair in the nest and measuring faecal lobe diameter (≥7.5 cm) for the silverback. The nest for the infant and its dam was confirmed by finding faecal materials of 2 distinctly different sizes; female (5.5 - 6.0 cm) and infant (≤3.0 cm) in the same nest. Only one adult female had an infant at the time of the study. A fresh morning trail was also followed till contact was made with the gorilla family and samples taken whenever the subject animal was observed defecating. Faecal materials were inspected; and the general consistence noted. The faecal location was noted as either being on the trail (T) or in the nest (N). Time of taking samples was noted, and the sample time on the trail was further coded as morning (AM) or afternoon (PM). Firm faecal material was dissected using two pieces of wooden spatula and /or loose faeces were mixed and material picked. Five-ml cryogenic vials (# 430663; Corning Incorporated, Corning, NY 14831) were filled to the three-quarter mark and 99.7% ethanol (BDH lab supplies, Poole, BH15 1TD, England) added immediately after collection for preservation. The vials were capped, labelled and left at ambient temperature till they were transported to the Smithsonian Institution's Centre for Conservation and Research (CRC) for processing and storage as indicated above.

Over all, One-way Analysis Of Variance (ANOVA) was run to investigate the variation in FGM levels and the t-test was used to compare the pre Vs post treatment FGM levels. The assay procedural precision was denoted by intra-assay and inter-assay variations of 5.88 and 17.25%, respectively.

Faecal hormone extraction efficiency: The Feacal Glucocorticoid Metabolite (FGM) were extracted following standardized faecal extraction protocol (Wasser *et al*., 1994). Approximately 0.0250 g of dried sample was boiled in 6 ml of 95 % ethanol after addition of 100 μL ^{3}H tracer to monitor hormone extraction recovery rates, in a 96ºC water bath for 20 min, adding more alcohol if necessary. The suspension was vortexed, centrifuged at 2500 rpm for 15 min. The supernatant was decanted into another glass tube and dried down under air. This was then reconstituted in 1 ml methanol before diluting with phosphate buffer (0.01 MPO_4, 0.14 M NaCl, 0.01% NaN_3, pH 7.4) at the rate of 1:1 (Lowland gorilla) and 1:4 (Mountain gorilla) for RIA, 1:64 and 1:1 for EIA in Mountain gorillas and Lowland gorilla respectively. Aliquots (25 μL) were used to measure ^{3}H tracer radioactivity counts per minute (cpm) in a Beta Counter (LS 6500 Multi-purpose Scintillation Counter; Beckman Coulter; Beckman Instruments Inc.) The percentage recovery of the ^{3}H-corticosterone with which samples were spiked before extraction ranged from 62.83 to 71.76% with an average of 68.46±0.64% (n = 1972). It is assumed that the unknown hormone metabolites in the gorilla faecal samples were recovered at approximately the same rate. Immunoreactive mass in faecal extracts was measured following protocols supplied by the assay kit manufacturers and expressed on a per gram dry weight basis.

Laboratory validation:

Parallelism and accuracy tests: Validation of utility of the RIA and EIA to detect FGM from extracts of gorillas was carried out by employing the principles of Midgley *et al*. (1969). A 1 mL pool of both Lowland and Mountain gorilla faecal extract was derived by combining 10 x 100 μL aliquots from random sample extracts and parallel displacement curves were obtained by comparing serial dilutions of 500 μL of the pooled extract and standard cortisol preparations. A dilution of 1:1 was determined to be optimal for Lowland gorillas and 1:4 for Mountain gorillas in the RIA assay, while optimal dilutions were 1:1 and 1:64 in the EIA assay for Lowland and Mountain gorillas respectively.

To test for quantitative recovery of FGM (accuracy) and demonstrate whether or not there are substances in the faecal extract that may interfere with the ability of assay to accurately assess faecal corticoid metabolites, half volumes (25 μL) of diluted low-mass faecal extracts of each species was spiked with half volume (50 μL) of standard steroids (12.5 - 1000 ng/mL). An additional 25 μl steroid diluent was added to each tube to bring the final assay volume to 100 μl, and the mass of the unknown sample or 'background mass' was determined by combining 25 μl of the low-mass sample with 75 μl of steroid diluent. The mixtures was assayed using ICN corticosterone RIA (ICN Biomedicals, Inc., Diagnostics Division, 3300 Hyland Avenue, Costa Mesa, CA 92626) and/or HRP- Munro cortisol EIA (In house Assay Services Unit, National Primate Research Centre, University of Wisconsin, Madison), following the manufacturer's protocol. After subtracting background mass, a plot was made of the measured immunoreactive mass (ng/mL) against the known mass (ng/mL) of standard steroid originally added to each tube and used regression analysis to explore linearity and slope of the curve generated.

High Performance Liquid Chromatography (HPLC): HPLC was carried out to confirm existence of FMG in gorilla faecal supernatant. Exploration was made on use of the following two solvent gradients including 100% Isocratic (MeOH, 58%; distilled H2O, 40%; 0.5 N KH2PO4, 0.4%; 5 N NaOH, 0.33%; pH 4.5) at a flow rate, 0.75 mL/min over 90 min protocol used in San Diego Zoo lab, and 20-80% Methanol at a flow rate 1 mL/min over 80 min protocol used at the Smithsonian Institution's Conservation and Research Centre (CRC); National Zoo. Three high-peak (Lowland gorilla) and 6

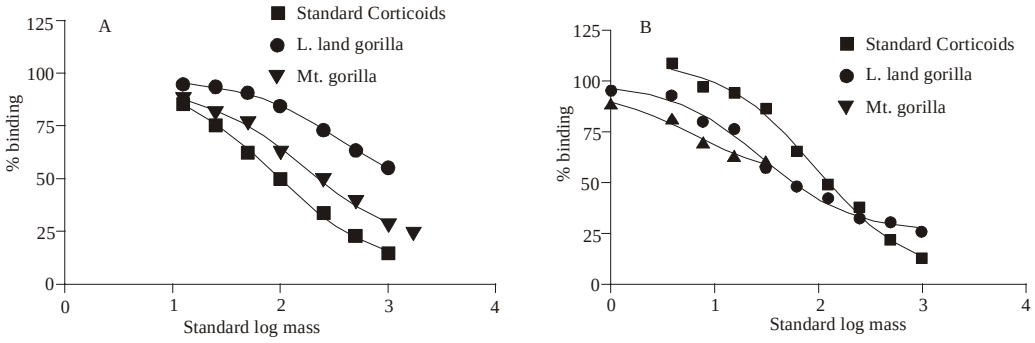

Fig. 1: Parallelism test on gorilla faecal glucocorticoid extracts; (a) RIA, (b) EIA

high-peak (Mountain gorilla) samples were extracted as described above, dried down after pooling supernatant for each subspecies and re-constituted in 1 mL of PBS buffer (pH 5.0), vortexed with Vorter-type 37600 mixer (MaxiMix I) followed by filtering. The filtrate was dried down and reconstituted by directly spiking it with the ^{3}H corticoid tracers (Cortisol;52 μL, Corticosterone; 35 μL and Deoxcorticosterone; 200 μL) allowing for approximately 8000 cpm/tracer of the stock steroids in 55 μL volume of the suspension. A 55 μL aliquot of the suspension was injected onto the C18 column (5 μm particle size, Varian, Inc., USA). Radioactivity in 50 μL of the sample suspension and that of 100 μL of each fraction eluate was determined in a Beta counter, correction factors were considered and data used to calculate the % recovery of the C18 column.

To measure immunoreactivity in each fraction, the remaining eluates (650 μL/tube; Isocratic gradient) were evaporated, reconstituted in steroid diluent (125 μL), which resulted in concentrating the steroid mass in each fraction by more than 5-fold (650:125), while the remaining eluates (900 μL/tube; 20-80% Methanol gradient) were also evaporated and reconstituted in steroid diluent (100 μL), which resulted in concentrating the steroid mass in each fraction 9-fold (900:100). Eluate suspension were assayed in singlet (50μL/fraction) following assay kit protocol. The corrected FGM mass per fraction (pg/fraction) and radioactivity (cpm/fraction) were plotted (lines on 2 axes) against the fraction interval and compared the FGM elution profile with the co-eluting radiolabelled corticoid standards.

RESULTS

Laboratory validation:

Parallelism and accuracy tests: The glucocrticoid metabolites in Lowland and Mountain gorilla faecal samples behaved in a similar manner as known standard corticoids when serially diluted (Fig. 1a, b). For

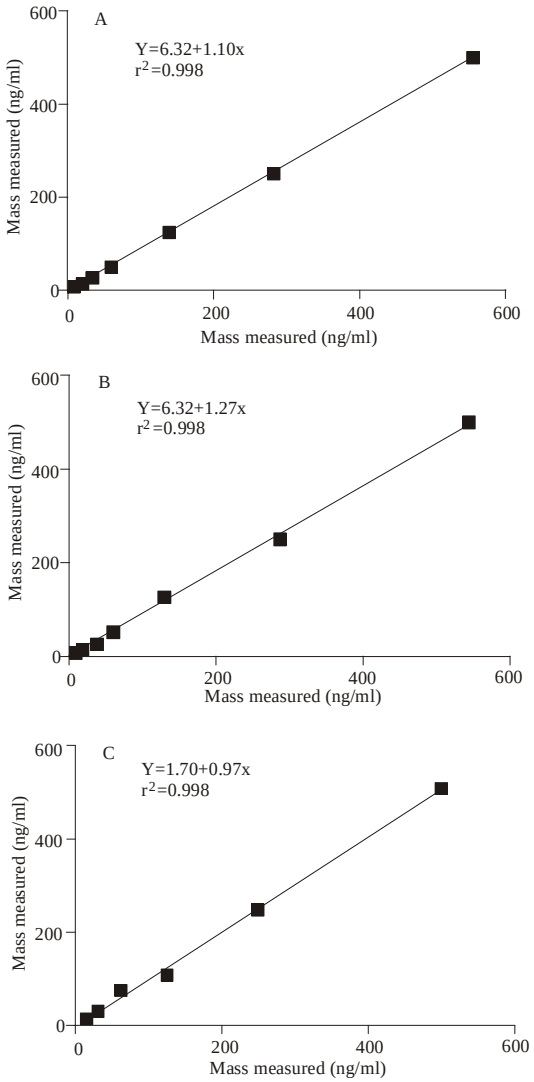

Fig. 2: Regression curve of gorilla faecal glucocorticoid assay accuracy: (a) lowland; RIA, (b) Mountain gorilla; RIA, (c) Mountain gorilla; EIA

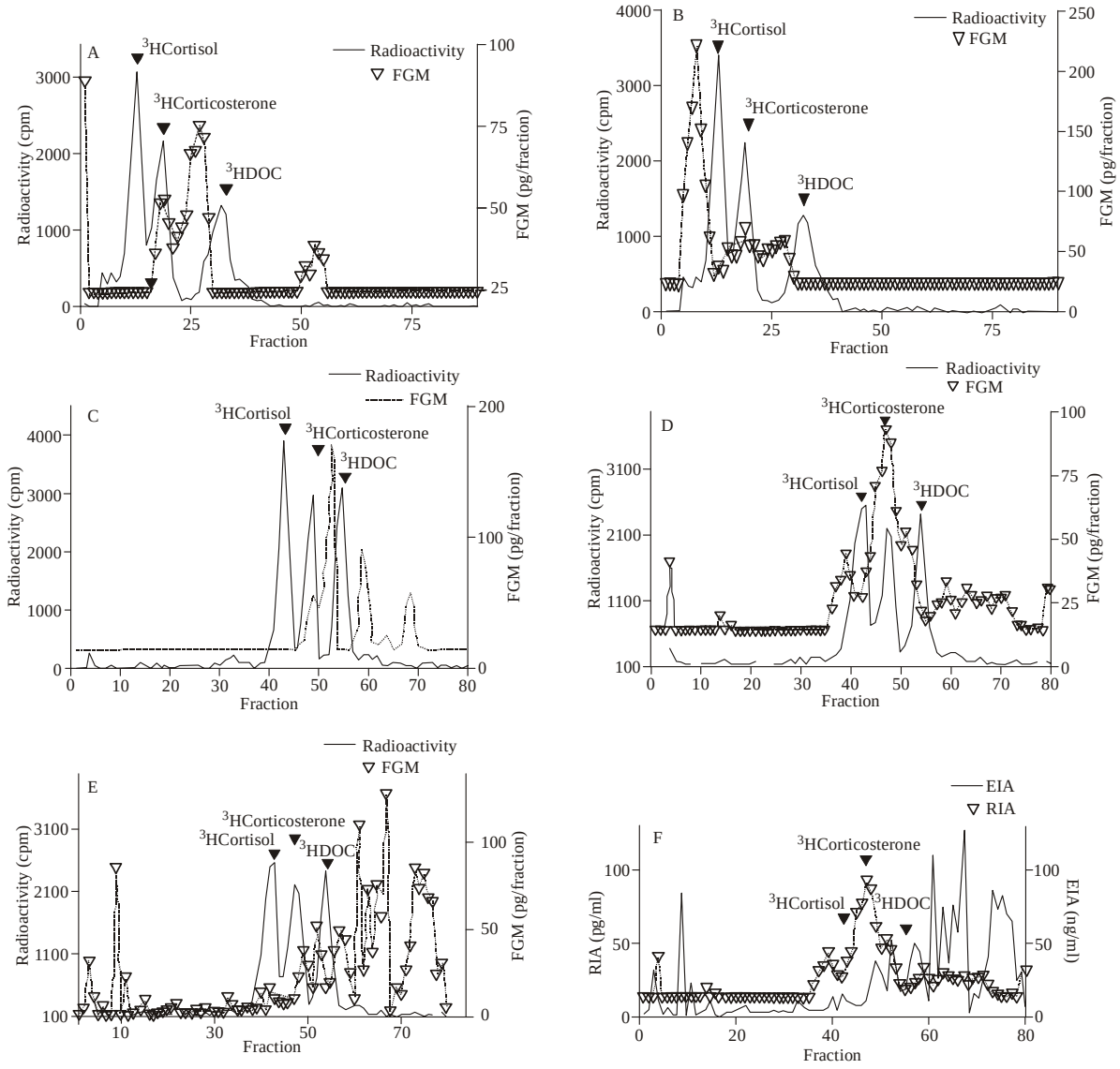

Fig. 3: High Pressure Liquid Chromatography of gorilla faecal glucocorticoid metabolites using 100% isocratic and 20-80% MeOH gradients: (a) Lowland gorilla; Isocratic, (b) Mountain gorilla; Isocratic, (c) Lowland gorilla; MeOH, (d) Mountain gorilla; MeOH, (e) Mountain gorilla; MeOH; EIA, (f), Mountain gorilla; MeOH; RIA & EIA.

Mountain gorilla, unknown sample dilutions between1/2 to 1/4 (RIA); 1/2 to 1/64 (EIA) bound at 50% on the standard curve suggesting that 1:4 and 1:64 are appropriate dilution factors for quantifying immunoreactive FGM using the ICN RIA and Munro EIA respectively. On the other hand, to measure FMG in Lowland gorilla by both assays required more concentrated material at a dilution factor of 1:1

Quantitative recovery of FGM was approximately 100% in both subspecies, indicating insignificant interference with the ability of the RIA to measure the corticoids in both the Lowland and Mountain gorilla faecal extracts (r^2 = 0.998; Fig. 2a, b). Similarly, no interference was found with the ability of the EIA to detect FGM in the Mountain gorilla (r^2 = 0.997; Fig. 2c)

Gorilla faecal corticoid chromatography:

Lowland and Mountain gorilla faecal corticoid HPLC using 100% Isocratic solvent gradient; RIA: In Lowland gorilla chromatography of FGM using Isocratic solvent gradient achieved a column (C18) recovery of 78.41%, and revealed a small proportion (10.3%) of total corticoid immunoreactivity which co-eluted with labelled corticosterone. The major immunoreactive peak (17.30%, fractions 22-30) and a minor peak (9.32% fraction 49-56) were made of metabolites that are less polar than

Table 1: Lowland gorilla mean FGM increase after ACTH injection

Assay	Time post-ACTH (hours)	Calculated F	df	Mean±SEM (ng/g)	p-value
RIA	24	16.20	2	4789.12±256.98	0.056
	48	0.95	6	4680.40±651.47	0.370
	72	5.57	14	5537.02±450.79	0.033
	96	5.37	20	5307.02±318.27	0.032
	120	3.02	24	5014.10±292.61	0.960
EIA	24	16.94	3	150.16±6.64	0.054
	48	8.68	9	134.30±8.05	0.020
	72	9.66	15	144.40±8.18	0.007
	96	11.76	20	149.46±7.37	0.003
	120	8.45	24	144.85±7.20	0.008

corticosterone (Fig. 3a). On the other hand, the C18 column achieved 100% FGM recovery under Isocratic gradient in Mountain gorilla, and revealed a small proportion (16.10%) of total corticoid immunoreactivity which co-eluted with labelled corticosterone. The major peak (29.32%, fractions 4-12) was made up metabolites that were more polar than the labelled cortisol (Fig. 3b). The difference in polarity of major immunoreactive peaks between the two gorilla subspecies may be a reflection of the intra-specific differences in steroid metabolites.

Lowland and mountain gorilla faecal corticoid HPLC using 20-80% Methanol solvent gradient; RIA: Chromatography of Lowland gorilla FGM using 20-80% MeOH solvent gradient achieved a column recovery of 60.28%, and revealed a small proportion (~10.74%) of total corticoid immunoreactivity, which co-eluted with labelled corticosterone though it was not quite separable from the major peak metabolites. The major peak was made up of immunoreactive metabolites (25.26%, fractions 49-54) which were less polar than the labelled corticosterone and more polar than the DOC (Fig. 3c). On the other hand, the C18 column achieved 100% recovery of Mountain gorilla FGM and revealed a major proportion (35.43%) of total corticoid immunoreactivity which co-eluted with labelled corticosterone (Fig. 3d), and at least one small peak of immunoreactive metabolites (10.36%, fractions 35-41) was detected that was more polar than cortisol. Also there were other small peaks that were inseparable from each other (25.90%, fractions 56-78) which were less polar than corticosterone.

Mountain gorilla faecal corticoid HPLC using 20-80% Methanol solvent gradient; EIA: Under the MeOH gradient, the EIA measured a small proportion (6.90 %, fractions 46-51) of total corticoid immunoreactivity in Mountain gorilla which co-eluted with labelled corticosterone. There were major multiple peaks that were inseparable from each other (fractions 52-80) consisting of metabolites which were less polar than all the 3 labelled hormones (Fig. 3e).

Comparison between Mountain gorilla immunoreactive metabolites measured by RIA and EIA; 20-80% MeOH: Comparison between ICN RIA and Munro EIA immunoreactive metabolites in Mountain gorilla FGM HPLC revealed that, the EIA detected immunoreactive metabolites that were relatively non-polar (i.e., greater retention times) compared to metabolites detected by the RIA (Fig. 3f). The major immunoreactive metabolite measured by the RIA co-eluted with maximum corticosterone radioactivity (fraction 47), whereas this was only a minor contributor to the overall immunoreactivity detected by the EIA. The ICN RIA manufacturer indicated that the kit cross-reacts with corticosterone at 100% cross-reactivity (ICN Biomedical, Inc. Taken together, the HPLC results indicated that separation of Mountain gorilla faecal corticoids using 20-80% MeOH solvent gradient and employing the non specific double-antibody ICN RIA offered the best and more specific arrangement for monitoring Mountain gorilla FGM. Though neither the major metabolites measured from HPLC fractions resulting from use of Isocratic solvent gradient, nor major metabolites measured by Munro EIA on fractions resultant from using 20-80% MeOH gradient co-eluted with peak radio labelled ^{3}H corticoid hormones, their measures can still be used to monitor gorilla stress if they can be proven to be physiologically relevant, even if the identity of specific measured metabolites are not yet characterised.

Physiological validation:

Feacal Glucocorticoid Metabolite (FMG) measures in lowland gorillas: Both the non-specific ICN RIA (ICN Corticosterone) and the EIA (Munro Cortisol EIA) were useful for quantifying the adrenal response to ACTH challenge (Fig. 4a). There was an initial rise in FGM within the first 12 h, however literature indicates that faecal corticoid excretion in a number of non human primates occurs between 24 to 72 h. Therefore this initial rise may or may not have been associated with adrenocortical response to the ACTH injection. The major increase peaked between 33-52 h (RIA), and between 52-74 h (EIA). The data was grouped into 24 h blocks (Table 1) and analysed with one-way ANOVA to find out when the increase in faecal corticoids was significantly different from the mean baseline concentration. The pre-ACTH mean corticoids measured by RIA was 3831.24±77.29 ng/g dry weight (n = 6). The post-ACTH mean concentration had a significant 45% increase ($p<0.05$) by 72 h up to 96 h, and there after the FGM

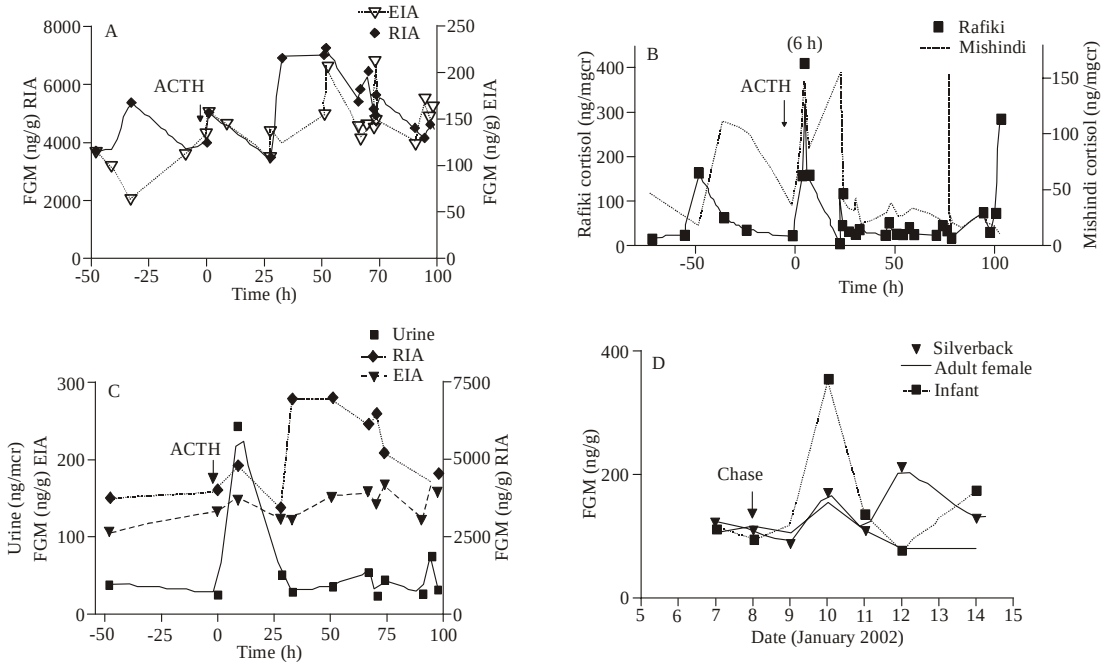

Fig. 4: Physiological relevance of RIA and EIA to measure gorilla response to stress: (a) Comparison of FGM measures after ACTH, (b) Variation in urinary cortisol after ACTH, (c) Correspondence of urinary and faecal glucocorticoids after ACTH in Rafiki, (d) Adrenocortical response of three individuals after chasing a family of Mountain gorillas from fields

levels decreased towards the pre-ACTH values ($p>0.05$). However the mean corticoid levels (7079.38±100.66 ng/g) within the time period (33-52 h) when highest values were recorded (in which 48 h falls); showed approximately a 2-fold increase from the mean baseline hormonal levels. Therefore it can be taken that the RIA was able to detect FGM increase at 48 h, the maximum time when detection of the first rise in adrenal response is expected in most non-human primates; though no sample was recovered at this exact time.

For the FGM measured by EIA, the mean baseline concentration was 104.16±9.64 ng/g (n = 6). The significant increase ($p<0.05$) was detected from 48 h. The mean levels were consistently higher by ~37% through 120 h post-ACTH. The maximum concentration (212.33 ng/g) was recorded at 74 h post-ACTH, which achieved a 2-fold increase from baseline mean levels.

The mean pre-ACTH FGM (3831.24±77.29 ng/g; RIA) was 37 times more ($p<0.05$, $t = 14.11$, $df = 5$) than mean pre-ACTH FGM levels measured by EIA (104.16±6.64ng/g). After ACTH injection, the mean response FGM levels measured by RIA (5222.97±285.52 ng/g) was 35 times more ($p<0.05$, $t = 16.83$, $df = 15$) than the mean response FMG levels measured by EIA (150.57±6.54 ng/g). This shows that there was consistence in the approximate ratio of FGM measured by each assay. Taken together, both the RIA and EIA were able to detect adrenocortical response to ACTH injection between 48 and 72 h, and are physiologically valid assays to monitor faecal corticosteroids in gorillas.

Apart from proving that the RIA and EIA can valuably be used to monitor faecal gorilla corticosteroids, the results also showed that the ACTH gel (150 IU Acthar Gel, Rhone-Poulenc Rorer Pharmaceuticals, Inc., Collegeville, Pennsylvania 19034, USA,) together with 25 mg of cortisol (combined with 10 mg of deuterium-labelled cortisol) is effective in stimulating Lowland gorilla adrenal cortex.

Urinary Cortisol measures in lowland gorilla: Though urinary corticoid baseline concentrations varied considerably between individuals, all test subjects except the Mexico City Zoo male; showed increased urinary corticoids in response to ACTH administration. The urinary corticoids peaked between 1 and 6 h post-ACTH (Fig. 4b) and there after decreased towards the baseline values.

The mean post ACTH urinary cortisol for Rafiki (286.4±125.1 ng/mgcr) in the first 6 hours was 5-fold more ($p<0.05$, $F = 10.15$, $df = 7$) than the pre-ACTH mean levels (55.3±23.13 ng/mgcr). There was no significant difference ($p>0.05$, $F = 4.09$, $df = 10$) between the baseline concentrations and the mean level (179.56±61.0 ng/mgcr) excretion by 24 h after ACTH

injection. The urinary cortisol peak in Rafiki seem to have corresponded with the initial minor peak observed in FGM (Fig. 4c).

Though there was observed increase in urinary excretion profile of Mishindi (Fig. 4b), the mean pre-ACTH injection concentration (64.53±15.68 ng/mgcr) was not significantly different ($p>0.05$) from neither the mean cortisol excretion in the first 6 h (113.4±19.53 ng/mgcr) nor from the mean cortisol excretion within 24 h of injection(116.36±16.36 ng/mgcr). The lack of statistically significant difference in Mishindi compared to Rafiki, may be due to individual differences in steroid metabolism.

Field application of FGM measures in free-ranging Mountain gorillas: For field application, samples from 3 individuals of Mubale free-ranging gorilla family were assessed with ICN RIA kit. Results revealed an increase in FGM excretion between the 2nd and 3rd day (Fig. 4d) after the family was chased. The mean pre-chase FGM concentrations for the silverback (107.36 ± 10.0 ng/g), adult female (108.41±3.62 ng/g) and infant (108.41±6.70 ng/g) were not significantly different ($p>0.05$) from respective post-chase mean FGM levels (154.86±22.60 ng/g, 131.55±23.67 ng/g, 185.79±59.87 ng/g) even though these post-chase mean FGM levels marked approximately 44, 21, and 71% increase, respectively over the pre-chase values. The lack of statistical difference between the pre-chase and post-chase mean faecal corticoids should however be interpreted with caution as there other uncontrollable factors in the field setting that can affect effectiveness of an environmental stressor in eliciting adrenal response.

DISCUSSION

Laboratory validation: Reimers *et al*. (1981) stated that immuno assays must be re-validated for each new species or medium a fact which has been reinforced by Heistermann *et al*. (2006). While Czekala *et al*. (1994) validated Cortisol RIA assay for measurement of urinary corticoids in gorillas, in the current study the non specific corticosterone RIA and cortisol EIA were validated for the measurement of FGM in the free-ranging Mountain gorillas using the captive western lowland gorilla as a model for controlled experimental design to achieve physiological validation. Although not as many EIA tests as RIA were run on the samples due to limited resources, the results of the current study are sufficient to draw useful conclusion that adds to the wealth of knowledge in application of the non-invasive assay technologies in wildlife.

Interestingly, for faecal corticoids to be detected by both assay kits in the Lowland gorilla it required more concentrated faecal extracts (1:1) compared to Mountain gorillas whose dilution factor requirement for RIA and EIA were 1:4 and 1:64, respectively. This suggests that the Mountain gorilla extracts had more faecal corticoid metabolites which cross-reacted with the antibodies employed in these assay kits. However this may also point to the fact that there are intra-species variations in steroid hormone metabolism as noted by Reimers *et al*. (1981). On the other hand, the differences between the diet fed to captive gorillas and the diet taken in by foraging free-ranging gorillas has the potential to influence FGM measures due to the differences in the microbial flora and their subsequent effect on steroid hormone metabolism in the gut as suggested by Wasser *et al*. (1993). Also Millspaugh and Washburn (2004) indicated that captive environment in itself has the possibility of influencing the amount of faecal glucorticoids excreted by captive animals compared to their free-ranging conspecific individuals.

Though limited, this study is one of the few which have attempted to compare utility of two assays on hormonal measures in a free-ranging species and to some extent comparing chromatography elution techniques used in 2 different labs. The varying results observed reinforce the currently recognized difficulties of comparing absolute values of faecal steroid metabolite studies among laboratories which may emerge from differences in the crossreactivities of antibodies used, in addition to serious concerns in selection of antibody and standards for immunoassays, and differences in extraction procedures and other technical procedures as pointed out by Palme *et al*. (1997). In this respect, the absence of immunoreactive metabolites co-eluting with authentic cortisol in this non-human primate ape (gorilla gorilla) may present difficulties of comparing with results of Whitten *et al*. (1998) who demonstrated that cortisol could provide a valuable faecal marker for monitoring adrenocortical activity in another ape (chimpanzee; Pan troglodytes), and proposed that it could find application in other ape species like gorillas. In other primates, Bahr *et al*. (2000) who used an EIA system (cortisol, corticosterone, 11-Oxoetiocholanolone) found out that radioactivity eluting at cortisol positions was low in marmoset (Callithrix jacchus), and almost absent in both the macaques (Macaca fascicularis) and chimpanzee (Pan troglodytes). The latter authors noted that the results of Whitten and co-workers on the chimpanzee study were a reflection of using a single highly specific antibody in a sensitive RIA system which had an activity range of ten times lower than the commercial assays. However Cavigelli (1999) also found out that faecal cortisol was useful in monitoring adrenocortical activity in lemurid primates (Lemur catta). Despite these differences, which again can emerge from either species and individual animal differences, or differences in laboratory procedures; the results of the current study on the gorilla may be in line with the conclusion made by Bahr and co-workers, that cortisol is not excreted in significant

quantities in primate faeces as had also been shown for other species such as cats (Graham and Brown, 1996), and sheep (Palme and Möstl, 1997).

The HPLC confirmed the validity of using the non specific ICN RIA and Munro EIA to detect trends in faecal steroid metabolite profiles in gorillas even if the specific metabolites are not yet characterized, but were able to predict physiological processes. The RIA was able to measure immunoreactive FGM in Mountain gorilla whose peak co-eluted with maximum standard corticosterone radioactivity. In view of the fact that the Non-specifc ICN corticosterone kit which was used cross reacts with 100% corticosterone (ICN Biomedicals, Inc), the current results suggest that this ICN RIA can valuably be used as a more-less specific assay for measurement of faecal corticosterone in free-ranging Mountain gorillas and would not need any modification. The only other RIAs ever used on faecal extracts of apes was a modified Pentex Direct 125I cortisol kit (Santa Monica, Ca) which has a cross reactivity of <0.03% for corticosterone (Whitten *et a1*., 1998) and measured faecal cortisol as noted above.

The immunoreactive metabolites measured by Munro EIA in Mountain gorilla appeared in distinctive multiple peaks between fraction 52 and 80, behaving more-less like the study of Goyman *et al*. (1999) which used an EIA system to measure faecal corticoids in spotted hyenas (*Crocuta crocuta*). In order to attain better results it would be advisable to employ the concept of group-specific EIAs that cross-react with a diversity of faecal corticoids as was suggested by Schwarzenberger *et al*. (1996b), Palme *et al*. (1997) and later described by Bahr *et al*. (2000) who employed an 11-oxoetiocholannolone EIA (a group specific EIA) for non-invasive assessment of adrenal activity in new and old world monkeys, and great apes. Establishment of such an assay system is important in view of the fact that EIAs would be more user friendly in the Mountain gorilla range countries which are looking for technology transfer that can be incorporated in health monitoring programs of this highly endangered species; and are as yet less equipped to manage radioactive wastes that would accumulate from use of RIAs. In addition, RIA procedures are less likely to be methods of choice when the non-invasive technologies become developed to the level of being field based.

Physiological validation: A 5-fold increase in gorilla urinary corticoid metabolites was detected between 1 and 6 hours post-ACTH challenge, this falls within the scientifically accepted view that most urinary steroid excretion appears in less than 12 h after the stimulation of the adrenal cortex, depending on the animal species Monfort *et al.* (1990, 1997). The corresponding 2-fold increase in faecal corticoids was detected between 33 and 52 h post injection as measured by both the RIA and EIA, which also agrees with the time range (30-52 h) of faecal glucocorticoid excretion in other primates including marmoset, macaques and chimpanzee (Bahr *et al*., 2000) and black howlers (Martínez-Mota *et al*., 2008). Also the current results are comparable to the range of 36 to 48 h which have been reported for peak excretion of ovarian steroids in primates (Ziegler *et al*., 1989; Shideler *et al*., 1993; Wasser *et al*., 1994). However it is important to realize that there can be slight alteration in the specific timing for detecting peak excretion since it is recognized that there exists within species and inter species differences in steroid hormone metabolism and excretion (Reimers *et al*., 1981), and that the lag time does also vary with different steroids for a given species.

It has been suggested that the delay of faecal glucocorticoid excretion may correspond to food transit time from the duodenum to the rectum for domestic livestock (Palme and Möstl, 1997), domestic cats (Graham and Brown, 1996). Goyman *et al*. (1999) used the gut transit time to estimate the faecal glucocorticoid excretion lag-time in spotted hyenas (*Crocutta crocutta*). In the same way, the faecal glucorticoid lag-time of 1.25 to 3 days observed in the current study is suggestive of the gut transit time in the gorillas, though specific experimental designs are needed to provide more confirmatory information.

In free-ranging Mountain gorillas, experimental designs can't be carried out to stimulate increased adrenal response. However, there are incidental activities that are highly suspected to induce increased adrenal activity. Gorillas often range outside the park, including instances when gorillas were observed raiding banana plantations and were chased back by humans into the park. The observed increase in FGM excretion between 2nd and 3rd day after the family was chased from banana plantation falls within the FGM excretion lag-time range of 1 to 3 days documented by various researchers in a number of other non human primates including *Sanguinus oedipus* (Ziegler *et al*., 1989), *Macaca mulatta* (Stavisky *et al*., 1992), *Macca fascicularis* (Shideler *et al*., 1993), *Callithrix jacchus* and *Saguinus fuscicollis* (Heistermann *et al*., 1993), *Papio cynocephalus* (Wasser *et al*., 1994), *Cercocebus torquatus atys* (Whitten and Russel, 1996), *Pan troglodytes* (Milton and Demment, 1998), *Nycticebus coucang* and *Lemur catta* (Perez *et al*., 1998), and *Alouatta pigra* (Martínez-Mota *et al*., 2008).

Though there was no statistical difference between the pre-chase and post-chase mean faecal corticoids, this may not be surprising, considering that significant difference between the baseline and post-ACTH injection mean FGM levels in Lowland gorillas was detected only after attaining a 2-fold increase, which level was not attained in the chasing incidence on Mountain gorillas. Therefore these results should be interpreted with caution as there are may be a number of factors in the field setting such as chasing intensity, method and duration of chasing

that can affect the effectiveness of chasing as a stressor. Nonetheless, the results on free-ranging Mountain gorilla in the current study provides another proof of the physiological validity of the ICN RIA in monitoring adrenal activity in this species, and adds to the list of about 22 free-living mammal species in which non-invasive endocrine monitoring has been described or has found field application (Monfort, 2001).

CONCLUSION

In conclusion, the 150 IU Acthar Gel is an effective stimulant of adrenal cortex of the gorilla and the resultant Feacal Glucocorticoid Metabolite excretory lag-time is between one and three days. Both the ICN RIA and Munro EIA are able to detect a cause-effective relationship and can be used to measure faecal markers of stress in gorillas. Mountain gorilla FGM co-eluted with peak immunoradiactivity to corticosterone making the ICNRIA a better assay of choice to non-invasively assess the environmental and social impact on adrenal activity in this species.

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